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Loss of ER retention and sequestration of the wild-type ELOVL4 by Stargardt disease dominant negative mutants

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Purpose: Mutations in *ELOVL4*, a member of the fatty acid elongase (*ELO*) family, are responsible for autosomal dominant Stargardt-like macular degeneration. The specific role of ELOVL4 in photoreceptors and the degenerative events induced by dominant ELOVL4 mutations are not well understood. As a first step to identifying possible mechanisms contributing to cellular dysfunction, we transfected HEK293 and COS cells with fluorescent-labeled wild-type and mutant ELOVL4 constructs. Effects of mutant ELOVL4 on interaction with wild-type protein were examined in this in vitro model.

Methods: Wild-type and mutant ELOVL4 proteins including ELOVL4 truncation (270X, a truncated ELOVL4 protein at amino acid position 270) and ELOVL4 5 bp deletion (5bp-del) and ELOVL4 (5A, substituting the ER retention signal, KAKGD, with a five alanine amino acid tract) were expressed as EGFP or DsRed fusion proteins. Cellular localization of these proteins was examined by fluorescence microscopy. ELOVL4 protein aggregates were measured by co-immunoprecipitation and by sucrose gradient centrifugation followed by immunodetection with western blots. To study cellular status of cells expressing mutant ELOVL4 proteins, transfected cells were examined for upregulation of Bip and CHOP, markers for the unfolded protein response (UPR) by western blotting.

Results: ELOVL4 mutants were not retained within the ER but were rather mislocalized and formed aggregates. Importantly, when cotransfected with wild-type ELOVL4, the mutants bound to and sequestered the wild-type protein into the aggregates. Expression of ELOVL4 mutants also induced UPR as evidenced by Bip and CHOP expression.

Conclusions: Using this in vitro cell system, we have identified alterations in wild-type ELOVL4 protein localization, aggregate formation, and the induction of cellular stress by the ELOVL4 mutants. We propose that "inactivation" of the wild-type ELOVL4 protein through sequestration to a non-ER compartment by ELOVL4 mutants may play a role in cellular dysfunction.

Stargardt-like macular degeneration (STGD3; OMIM 600110) is an autosomal dominant form of juvenile macular degeneration characterized by decreased visual acuity, macular atrophy, and extensive flecks [1,2]. The disease shares some similarity with age-related macular degeneration (AMD), the leading cause of legal blindness in the elderly in developed countries, including abnormal accumulation of lipofuscin in the retinal pigment epithelium (RPE) and degeneration of RPE and macular photoreceptors. The disease-causing gene, ELOVL4, has been identified, however the function of the encoded protein is not known, nor is it understood why the retina is uniquely sensitive to disease-causing mutations in this gene. ELOVL4 encodes a 314 amino acid protein with sequence and structural similarities to the ELO family of proteins [3]. The ELO family of proteins is involved in the elongation of long chain fatty acids and is characterized by multiple putative membrane-spanning domains, a histidine cluster motif (HXXHH) essential for the enzymatic activity [4,5], and an ER retention signal (KXKXX) [6]. Therefore, based on similarities of ELOVL4 gene structure to the ELO family, it has been proposed that the protein may play a role in fatty acid elongation within the ER [7-10]. The importance of this protein is underscored by the fact that ELOVL4 is strongly conserved throughout vertebrate species [11,12].

Three *ELOVL4* mutations causing juvenile macular degeneration have been identified thus far. The first mutation is a five base pair deletion starting at position 790 of the open reading frame (790-794delAACTT, 5bp-del) [3] causing a frame-shift and a premature stop codon. The second mutation contains two 1 base pair deletions, 789delT and 794delT [13], which produces a frame-shift nearly identical to the five base pair deletion. We recently reported the third mutation, a transversion of C->G at position 810 causing a stop codon at amino acid 270 (270X) [14]. Interestingly, all three mutations occur around the same location and subsequent deletion of the C-terminus includes loss of the putative dilysine ER retention signal.

These ELOVL4 mutations induce a dominant effect over the wild-type allele resulting in Stargardt macular dystrophy. Previous studies demonstrated that unlike wild-type ELOVL4, the mutants do not localize to the ER [9,10]. Although no his-

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topathologic studies on postmortem specimens of affected STGD3 patients have been reported, our recent results demonstrate lipofuscin accumulation, electrophysiological changes, and photoreceptor degeneration in mutant *ELOVL4* transgenic mice in a manner that is closely related to the human phenotype [15]. In this study, we co-expressed mutant and wild-type ELOVL4 proteins in cell culture and examined effects of the mutant over the wild-type protein with respect to cellular localization. We demonstrate that mutant ELOVL4 altered proper ER localization of wild-type ELOVL4 and sequestered the wild-type and mutant proteins into dense aggregates.

Aggregated, misfolded proteins have been shown to bind chaperones and activate the unfolded protein response (UPR) [16-18]. Based on formation of aggregates, we assessed effects of these mutants on induction of the UPR. ER stress was measured by altered expression of the resident ER molecular chaperones, Bip and CHOP [16]. We demonstrate that expression of mutant ELOVL4 led to activation of the UPR and subsequent ER stress.

METHODS

Reagents: Cell culture and transfection reagents were purchased from Gibco-BRL (Invitrogen, Carlsbad, CA). Polyclonal and monoclonal anti-EGFP antibodies and poly L-lysine were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Anti-DsRed monoclonal antibody and the ER specific marker pDsRed2-ER were purchased from Clontech. Bip (Clontech, Mountain View, CA), CHOP, and β-actin antibodies and Protein A/G plus agarose were purchased from Santa Cruz (Santa Cruz Biotech, Santa Cruz, CA). Chamber slides were purchased from Nunc (Nalge, Rochester, NY).

Generation of expression constructs: All vectors used in this study utilized a CMV promoter and expressed enhanced green fluorescent protein (EGFP) or DsRed/ELOVL4 fusion proteins. Construction of wild-type, 5 bp deletion, and 270X mutant *ELOVL4* expression vectors have been described elsewhere [9,14]. Briefly, forward and reverse primers (5'-CGC GGA TCC GCG ATG GGG CTC CTG GAC TC-3' and 5'-CGG GAT CCC GTT AAG CTG CTG CTG CTG CTC CAT TTT TCT GCT TTT TTC-3', incorporating *Kpn*I and *Bam*HI restriction sites) were used to amplify wild-type *ELOVL4* cDNA. The resultant PCR products were digested and cloned into the *Kpn*I and *Bam*HI sites in the pEGFPC1 vector purchased from Clontech (Clontech, Mountain View, CA). DsRed fusion constructs were generated by amplification of ELOVL4 using forward and reverse primers (5'-CAT GCC ATG GCC TCC TCC GAG AAG GTC-3'and 5'-GGG GTA CCC AGG

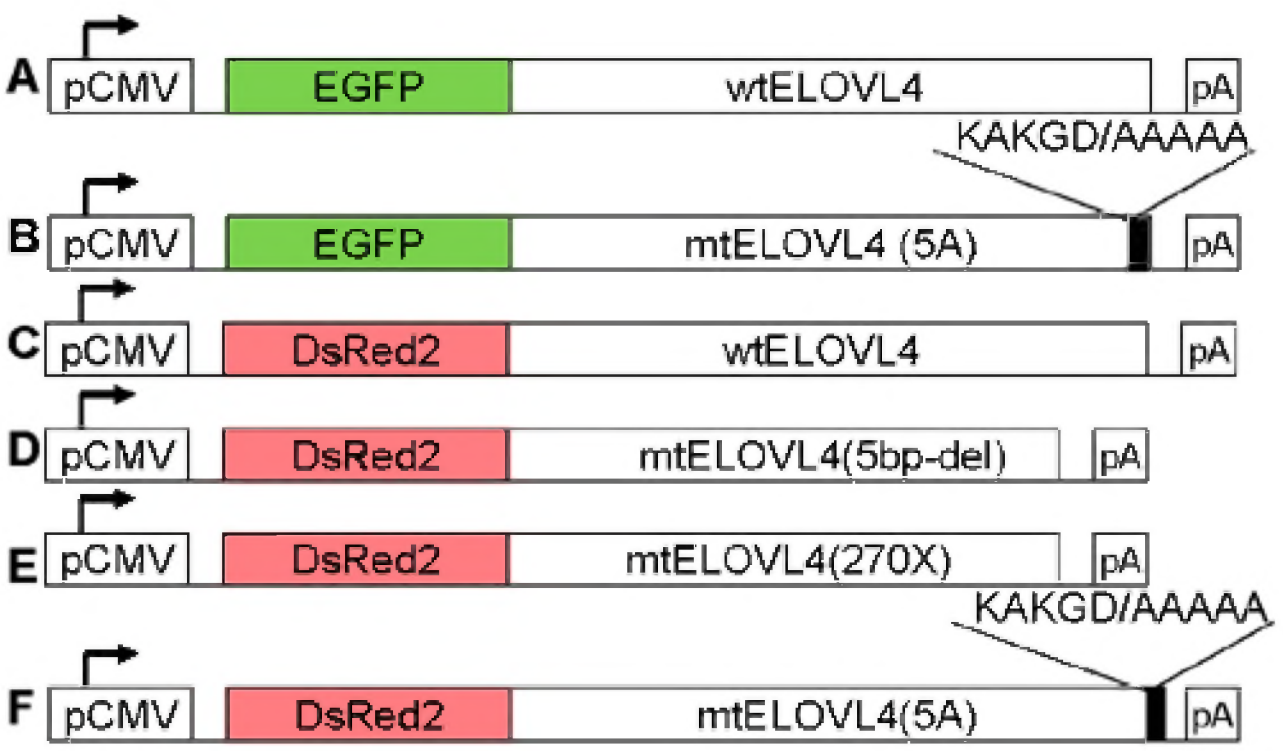

Figure 1. Nomenclature and diagrams of recombinant ELOVL4 constructs. **A**: EGFP and wild-type ELOVL4 fusion protein (wtEGFP/ELOVL4). **B**: EGFP fusion protein with the ER retention signal, KAKGD, replaced by AAAAA (mtEGFP/ELOVL4(5A)). **C**: DsRed2 and wild-type ELOVL4 fusion protein (wtDsRed/ELOVL4). **D**: DsRed2 and 5 bp deleted ELOVL4 mutant fusion protein (mtDsRed/ELOVL4(5bp-del)). **E**: DsRed2 and 270X ELOVL4 mutant fusion protein (mtDsRed/ELOVL4 (270X)) **F**: DsRed2 fusion protein with C-terminal ER retention signal, KAKGD, replaced with AAAAA (mtDsRed/ELOVL4(5A)). pCMV represents immediate early promoter from cytomegalovirus; EGFP represents enhanced green fluorescent protein; DsRed represents red fluorescent protein; pA represents polyadenylation signal from SV40 T-antigen; KAKGD/AAAAA represents dilysine motif replaced with alanine tract. All fluorescent proteins were fused in frame at the N-terminal end of the wild-type or mutant ELOVL4 amino acid sequence.

AAC AGG TGG TGG CGG C-3)' containing *Nco*I and *Kpn*I for insertion into the DsRed2 vector. Vector constructs and fusion proteins bearing a mutant amino acid sequence are designated with an "mt" prefix, while the wild-type counterparts bear a "wt" prefix. mtDsRed/ELOVL4(5bp-del), mtDsRed/ELOVL4(270X), and mtDsRed/ELOVL4(5A) were derived by in vitro mutagenesis as described in previous reports [9,14]. All recombinant plasmids were verified by DNA sequencing. A cartoon representation of the constructs and proteins is shown in Figure 1 illustrating the nomenclature of the fusion proteins used in the present experiments.

Transfection studies and image acquisition: COS-7 and HEK293 were used for all transfection studies. Details of transfection, fluorescence microscopy, and confocal microscopy have been described previously [9]. Briefly, cells were transfected at 50-60% confluence in 60 mm plates with 5 µg of respective recombinant DNAs using lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Recombinant protein expression was assayed at 24 h after transfection. To assess progressive expression of wild-type and mutant ELOVL4 proteins, transfected cells were examined by fluorescence microscopy at daily intervals post-transfection. No variability was noted and the data presented in this study were collected after approximately 24 h. Co-transfections with the ER specific marker pDsRed2-ER (Clontech, Mountain View, CA) were performed with wild-type and mutant ELOVL4 constructs to determine the subcellular localization of ELOVL4 proteins.

Immunoprecipitation: To investigate the interaction of wild-type EGFP/ELOVL4 and mutant DsRed/ELOVL4, co-immunoprecipitation experiments were conducted. Cells were first co-transfected with wtEGFP/ELOVL4 and mtDsRed/ELOVL4(5bp-del) recombinant fusion proteins and expression examined by fluorescence microscopy. Cells were collected 24 h after transfection and washed with PBS. Cell lysates were prepared with immunoprecipitation buffer (1% (v/v) Triton X-100 in 50 mM Tris-HCl buffer (pH 7.4), 0.15 M NaCl, 1 mM EDTA, and 0.1% (w/v) SDS containing protease inhibitor cocktail, Sigma). The polyclonal anti-EGFP antibody (2 µg of antibody per 1 ml reaction mixture containing 30 µl of Protein A/G agarose) was used for immunoprecipitation and proteins were separated by SDS-PAGE. A monoclonal anti-DsRed antibody (Clontech, Mountain View, CA) was used for western blot analysis.

Sucrose density gradient sedimentation: To investigate the interaction and aggregation of wild-type EGFP/ELOVL4 and mutant DsRed/ELOVL4, sucrose gradient sedimentation velocity experiments were conducted. HEK293 cells were cotransfected with wtEGFP/ELOVL4 and mtEGFP/ELOVL4(5bp-del), mtEGFP/ELOVL4(5A), or mtEGFP/ELOVL4(5bp-del) constructs. Cells were extracted in 10 mM Tris-HCl, pH 7.5, 0.25% Triton X-100, 2 µM digitonin, and protease inhibitors 24 h after transfection, and centrifuged at 4 °C for 30 min at 13,000x g. The supernatant (200 µl) was diluted with 50 mM sucrose in 5 mM HEPES (pH 7.2)/0.2 mM EDTA to a final volume of 300 µl and centrifuged on a 5 ml 5-20% (w/v) sucrose gradient for 18 h at 180,000x g in an SW50.1 rotor (Beckman Coulter, Fullerton, CA) [19]. Sucrose density gradient sedimentation was used to separate ELOVL4 and ELOVL4 aggregates based on expected differences in molecular weight and additional physiochemical properties. Therefore, higher molecular weight ELOVL4 aggregates are easily separated from the lower molecular weight, unaggregated ELOVL4 protein. To identify altered ELOVL4 sedimentation characteristics, 200 µl fractions and the pellet were collected and analyzed by western blotting.

Immunoblotting: For western analysis of Bip and CHOP proteins, the membranes were incubated overnight with anti-Bip and anti-CHOP (1:500) and β-actin antibodies (1:2000), and then probed with peroxidase-conjugated secondary antibodies (Amersham Biosciences Corp., Piscataway, NJ). An ECL detection kit (Amersham) was used according to the manufacturer's protocol. Tunicamycin exposure is known to upregulate CHOP in cell cultures [20] and, as such, tunicamycin treatment was used as a positive control for UPR induction. Cells were transfected with vector alone and treated with 10 or 20 µg/ml tunicamycin for 4 h at 37 °C, cells were harvested, and cell lysates used for western blot analysis.

RESULTS

Characterization of EGFP-ELOVL4 fusion proteins: Since an ELOVL4 antibody was not available for these studies we generated fluorescent ELOVL4 fusion proteins. Wild-type and mutant ELOVL4 were expressed as EGFP and DsRed fusion proteins (Figure 1 and [9]) to facilitate direct visualization. Similar results were obtained by Grayson et al. [21] using an ELOVL4/RIM epitope fusion protein. Furthermore, consistent with presence of a dilysine ER retention motif at the C-terminus of the protein, the ELOVL4/EGFP fusion protein colocalized with the ER specific marker, pDsRed2-ER (Figure 2B). Western blot analysis confirmed the synthesis of all ELOVL4 fusion proteins and single bands were visualized for each construct and shown in Figure 3 and Figure 4. Culture medium was examined by western blot analyses; no detectable protein was found (data not shown). Similar observations were found in both HEK293 and COS-7 cells. Wild-type ELOVL4 constructs were localized to the ER, and mutant ELOVL4 constructs were mislocalized as in our previous results [9], indicated by loss of co-fluorescent signal with the ER specific marker, pDsRed2-ER, (Figure 2B).

Dominant effect of mutant ELOVL4: Wild-type EGFP/ELOVL4 and mutant DsRed/ELOVL4 were co-expressed to examine effects of the mutant protein on wild-type cellular localization. Co-transfection of wild-type ELOVL4 constructs (EGFP/ELOVL4 and DsRed2/ELOVL4) showed a more diffuse cytoplasmic co-localized signal (Figure 2A, first row) residing predominantly within the ER as shown by co-immunolabeling with DsRed2-ER (Figure 2B). In all cells showing coexpression of the mutant (mtDsRed/ELOVL4(5bp-del), mtDsRed/ELOVL4(270X), or mtDsRed/ELOVL4(5A)) and wild-type proteins, immunolabeled proteins were co-localized in densely clumped aggregates (Figure 2A, arrows), which, upon co-expression of the DsRed2-ER marker, revealed loss of ER localization (Figure 2B). These results suggested

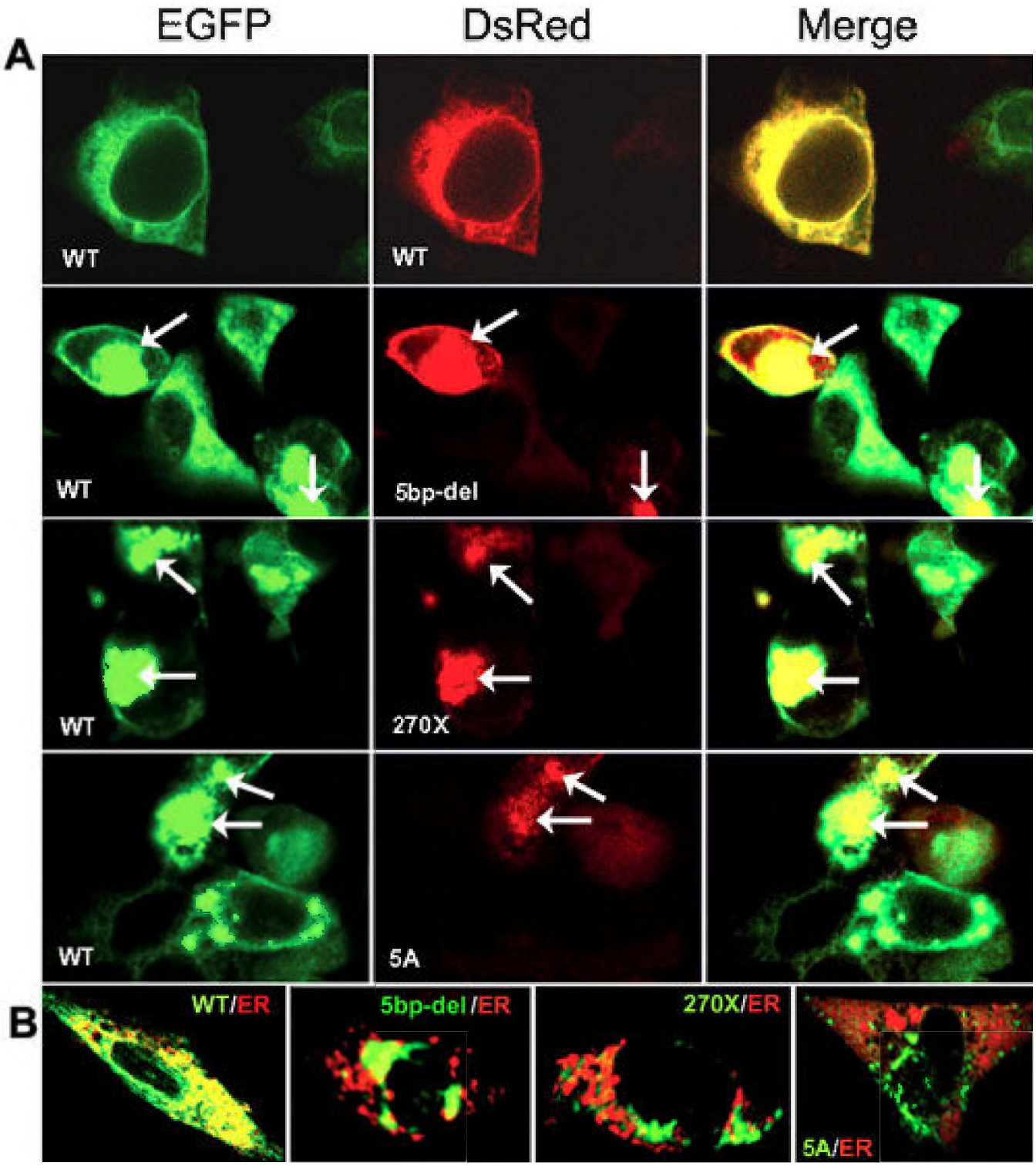

Figure 2. Localization of transfected ELOVL4 fusion proteins. Confocal images were recorded 24 h after transfection. **A**: wtEGFP/ELOVL4 was cotransfected (1:1) with wtDsRed/ELOVL4 or mutant DsRed/ELOVL4 constructs as indicated in each panel. The green color represents EGFP fused ELOVL4 wild-type protein, and the red color identifies the wild-type or mutant DsRed fused ELOVL4 proteins. When wild-type and mutant proteins were co-expressed, the overlapping (yellow) fluorescence signals were associated with aggregates (right panels). Note the clumped signals (both EGFP and DsRed) in the cells co-expressing mutant ELOVL4 protein (arrows). **B**: To visualize mislocalized ELOVL4 proteins, recombinant wtEGFP/ELOVL4 and mutant EGFP/ELOVL4 constructs were cotransfected with the ER specific marker, pDsRed2-ER. Mutant ELOVL4 protein signals (green) did not overlap with the ER protein marker signal (red).

that the mutant protein, showing loss of ER retention, could similarly affect normal distribution of the wild-type ELOVL4 protein.

Since the Stargardt mutations include deleted residues outside the ER retention domain, we wished to test whether the loss of this ER domain was responsible for altered protein localization. Therefore, the C-terminal ER retention domain was replaced with a tract of five alanines (and the mutant was named "5A"). We found a similar subcellular mislocalization between mtDsRed/ELOVL4(5A) and the other pathogenic mutants where there was no co-localization with an ER specific marker, pDsRed2-ER. This suggested that this shoprt domain was sufficient for mislocalization and, in coexpression assays, altered the normal ER localization of the wild-type protein.

Interaction of wild-type and mutant ELOVL4: The wtEGFP/ELOVL4 and mtDsRed/ELOVL4(5bp-del) proteins were co-expressed and co-immunoprecipitated with the EGFP antibody. The co-immunoprecipitated mutant proteins were subsequently identified by western blot using the DsRed antibody (Figure 3). Grayson et al. [21] recently reported similar results using an affinity column binding assay, but contrary to our results showed that wild-type ELOVL4 can associate with wild-type ELOVL4 protein. Using immunoprecipitation in this study, we saw no significant intramolecular association of the wild-type protein. This may have been due to less sensitivity using immunoprecipitation. However, we also saw no obvious aggregate formation in wild-type ELOVL4 transfection studies, suggesting that wild-type ELOVL4 protein interactions are not as extensive as that with the mutant ELOVL4 protein. This result demonstrates that the mutant and wild-type proteins associate when co-expressed.

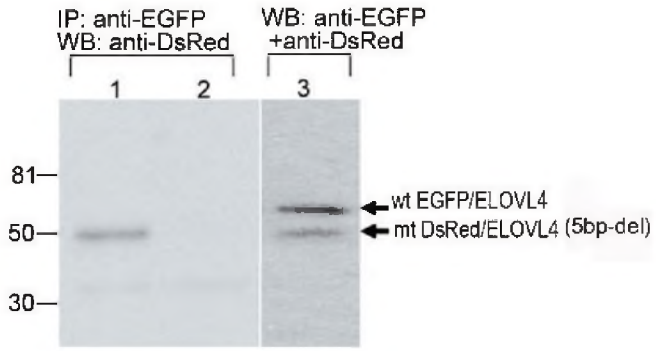

Figure 3. Western blots of co-immunoprecipitated wild-type and mutant ELOVL4. The EGFP antibody was used to precipitate wtEGFP/ELOVL4 and mutant ELOVL4 protein complexes. A DsRed monoclonal antibody was used for immunodetection of mtDsRed/ELOVL4 proteins. Lane 1: The EGFP antibody (specific for the wtEGFP/ELOVL4 protein construct) co-immunoprecipitated the mtDsRed/ELOVL4(5bp-del) protein. Lane 2: Untransfected control. Lane 3: Control showing expression of both proteins wtEGFP/ELOVL4 (upper band, about 63 kDa) and mtDsRed/ELOVL4(5bp-del, lower band, about 53 kDa) with co-transfection. IP represents immunoprecipitation; WB represents western blot; anti-EGFP represents antibody to enhanced green fluorescent protein; anti-DsRed represents antibody to red fluorescent protein; wt EGFP/ELOVL4 represents fusion protein containing the enhanced green fluorescent protein fused in frame to the N-terminal end of wildtype ELOVL4 protein; mtDsRed/ELOVL4(5bp-del); fusion protein containing the red fluorescent protein fused in frame at the N-terminal end of a mutant (C-terminal 5bp deletion) ELOVL4 protein.

To further demonstrate association of the wild-type and mutant ELOVL4 proteins (over that of wild-type alone), transfected cell lysates were separated on sucrose gradients, fractions were collected, electrophoresed, and immunolabeled on western blots. The wild-type protein sedimented with the lower molecular weight protein fractions (fraction numbers 15-18 in Figure 4). Conversely, MtDsRed/ELOVL4(5bp-del) and mtEGFP/ELOVL4(5A) formed high molecular weight aggregates (Figure 4). When coexpressed with mutant ELOVL4, the wild-type protein shifts to the higher molecular weight fractions (fraction numbers 1-10 in Figure 4A) similar to transfection of mutant ELOVL4 alone. These results suggest that wild-type and mutant proteins are in fact colocalized in cell aggregates. In addition, the ELOVL4(5A) mutant mimics the other ELOVL4 mutants (data not shown) in forming and sequestering wild-type ELOVL4 within higher molecular weight aggregates, thereby altering the proper localization of wild-type ELOVL4 proteins.

Induction of UPR associated proteins by mutant ELOVL4: The mutant ELOVL4 aggregates suggested that the mutant proteins may be misfolded. It is known that when ER is exposed to misfolded/unfolded proteins, cells respond by upregulating ER chaperones such as Bip/Grp78 and Grp94, and CHOP via the ER-kinase, PERK [22,23]. To investigate the involvement of a UPR to mutant ELOVL4 expression, we examined transfected cells for Bip and CHOP upregulation. We found that both Bip and CHOP protein levels were markedly increased by transfection of the mutant ELOVL4 (Figure 5B,C). These results were similar to that of the tunicamycin-treated group as a positive control for UPR activation (Figure 5E). Thus, mutant ELOVL4 protein-induced upregulation of the UPR proteins Bip and CHOP may initiate an ER stress response.

DISCUSSION

Mutations in ELOVL4 cause Stargardt-like macular degeneration, an autosomal dominant juvenile macular degeneration. The results of the present study suggested that ELOVL4 aggregates may induce some toxicity at photoreceptor and/or RPE cellular levels. This mechanism may be directly examined in photoreceptors and the RPE in vivo using our ELOVL4 transgenic mouse lines in future studies. Previous cell culture studies on the mutant ELOVL4 proteins have shown loss of ER localization. In this study, we found that mislocalization induced by the Stargardt causing ELOVL4 C-terminal mutants, ELOVL4(5bp-del), and ELOVL4(270X), can be reproduced by substitution of a five-alanine tract for the ER retention domain. Thus, the primary effect of these mutations most likely arises from loss of the ER retention signal. These results are in agreement with a previous study [10] showing that a transfected ELOVL4 construct, with specific deletion of the

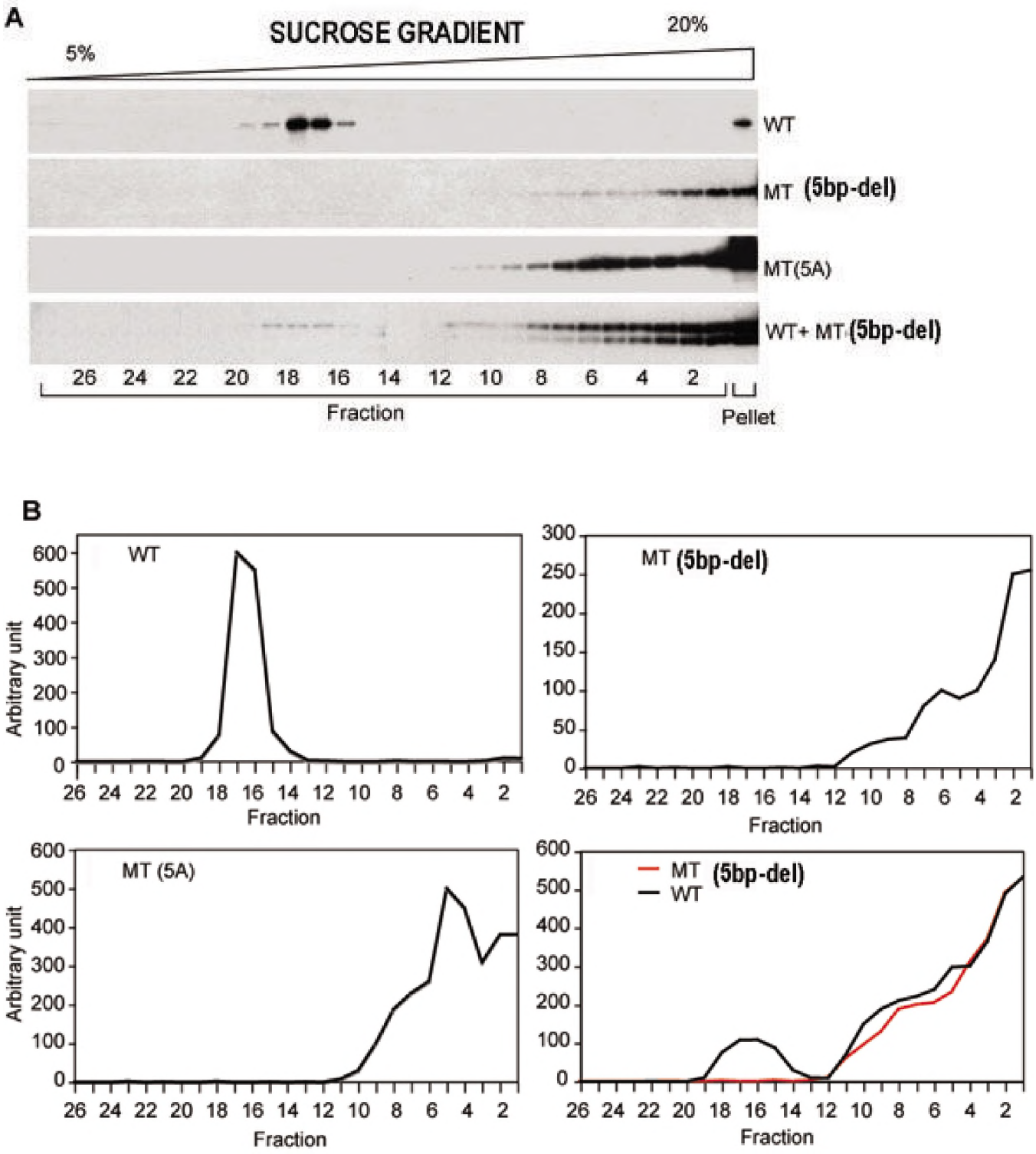

Figure 4. Sucrose density gradient sedimentation and densitometric analysis of ELOVL4 aggregates. **A**: Western blots show immunolabeled wtEGFP/ELOVL4 and mutant ELOVL4 protein aggregates fractionated by sucrose density gradients. Cells were transfected with wtEGFP/ELOVL4, mtEGFP/ELOVL4(5bp-del), or mtEGFP/ELOVL4(5A) or co-transfected with wtEGFP/ELOVL4 and mtEGFP/ELOVL4(5bp-del). wtEGFP/ELOVL4 signal was normally found in lower molecular weight fractions. The 5bp-del mutant and 5A mutant shifted to the lower fraction numbers indicating formation of larger molecular weight aggregates. Mutant ELOVL4 (mtEGFP/ELOVL4(5bp-del)) induced the wild-type protein (wtEGFP/ELOVL4) to shift to the higher molecular weight fractions. Co-transfected cell lysates showed strong immunolabeling in higher molecular weight fractions similar to that of single-mutant transfections. **B**: Fractionated ELOVL4 proteins (wtEGFP/ELOVL4, 5bp-del mutant, and 5A mutant) and co-transfected wtEGFP/ELOVL4 and mtEGFP/ELOVL4(5bp-del), measured by densitometry of western blots, are graphically represented.

last five amino acids, KAKGD, similarly exhibits no ER retention.

The role of ER stress in human pathogenesis has become a subject of great interest in the last few years. A growing number of neurodegenerative disorders such as Parkinson's, Huntington's, Alzheimer's, and prion diseases have been linked to common pathological features arising from aggregate formation and associated ER activation [24]. Protein aggregates in the form of neurofibrillary tangles, and cytoplasmic or nuclear inclusions induce the UPR, alter functional efficacy of the ubiquitin/proteasome system, and induce ER stress [18,25,26].

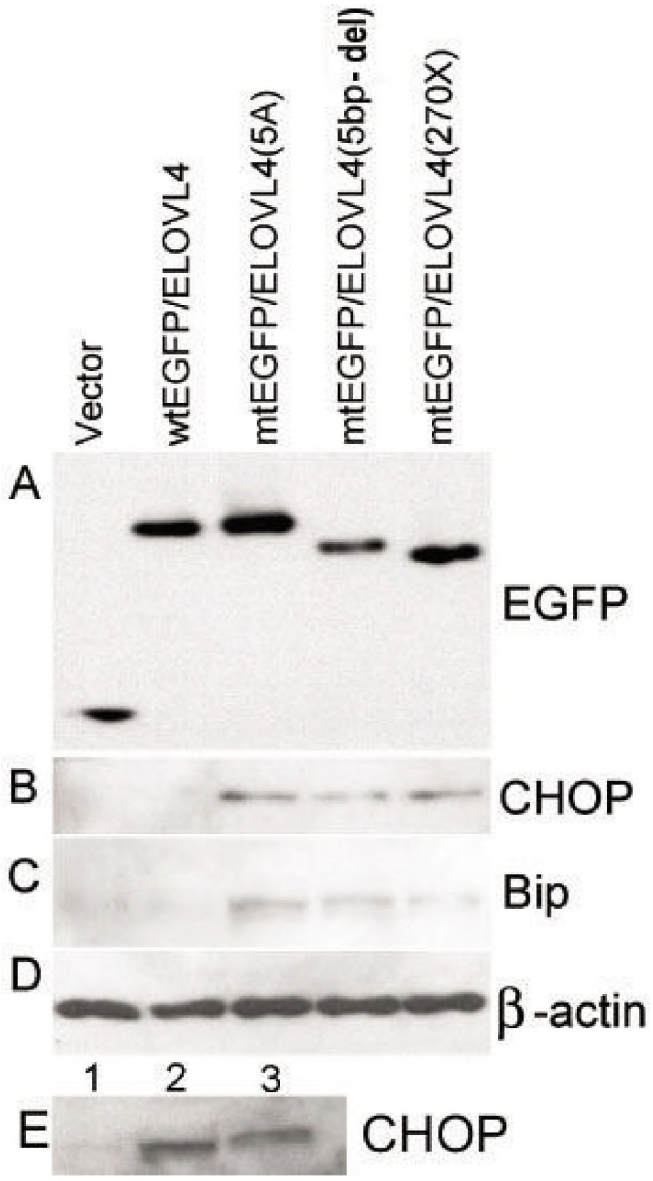

Figure 5. ELOVL4 mutants induce UPR response proteins, CHOP and Bip. Cell lysates were collected 24 h post-transfection with wild-type or mutant ELOVL4 constructs. **A**: Samples from left to right include: vector alone, mtELOVL4(5A), mtELOVL4(5bp-del), and mtELOVL4(270X). **B**: CHOP protein levels were induced by transfection of all ELOVL4 mutants, but not by overexpression of the wildtype ELOVL4 protein. **C**: Bip protein levels were similarly upregulated with mutant ELOVL4 expression. **D**: β-actin used as a loading control. **E**: Tunicamycin control experiment showed CHOP upregulation. Lane 1: Untreated. Lane 2: Tunicamycin treated (10 mg/ml). Lane 3: Tunicamycin treated (20 mg/ml).

An important finding of this study is that all ELOVL4 mutants inhibited wild-type ELOVL4 ER localization and redistributed the protein into aggregates. While expression of mutant ELOVL4 alone was sufficient for aggregate formation and induction of the UPR, corresponding mislocalization of the wild-type protein may have further consequences. Mislocalization may serve to inhibit the normal function of the wild-type protein, in essence, inducing a null ELOVL4 phenotype. While these results revealed a possible dominant negative mechanism for mutant ELOVL4-induced cellular dysfunction, corresponding effects in photoreceptors and subsequent consequences on the RPE need to be addressed. This may be directly examined in vivo using transgenic mice that express mutant ELOVL4 [15], and the mouse disease closely resembles human Stargardt macular degeneration.

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